Effects of Sea Animal Colonization on the Coupling between Dynamics and Activity of Soil Ammonia-oxidizing Bacteria and Archaea in Maritime Antarctica

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Abstract

The colonization of a large number of sea animal, including penguins and seals, plays an important role in the nitrogen cycle of the tundra ecosystem in coastal Antarctica. However, little is known about the effects of sea animal colonization on ammonia-oxidizing archaea (AOA) and bacteria (AOB) communities involved in nitrogen transformations. In this study, we chose active seal colony tundra soils (STS), penguin colony soils (PTS), adjacent penguin-lacking tundra soils (PLS), tundra marsh soils (MS), and background tundra soils (BS), to investigate the effects of sea animal colonization on the abundance, activity, and diversity of AOA and AOB in maritime Antarctica. Results indicated that AOB dominated over AOA in PTS, STS, and PLS; whereas AOB and AOA abundances were similar in MS and BS. Penguin or seal activities increases the abundance of soil AOB amoA genes, but reduced the abundance of AOA amoA genes, leading to very large ratios (1.5 × 10^2 to 3.2×10^4) of AOB to AOA amoA copy numbers. Ammonia oxidation rates were significantly higher (P = 0.02) in STS and PTS than in PLS, MS, and BS, and were significantly positively correlated (P < 0.001) with AOB amoA gene abundance suggesting that AOB are more important in the nitrification in animal colony soils. Sequence analysis for gene clones showed that AOA and AOB in tundra soils were from the *Nitrososphaera* and *Nitrosospira* lineages, respectively. Seal or penguin activities led to the predominant existence of AOA phylotypes related to *Nitrososphaera* cluster I and AOB phylotypes related to *Nitrosospira* clusters I and II, but very low relative abundances in AOA phylotypes related to cluster II, and AOB phylotypes related to cluster III and IV. The differences in AOB and AOA community structures were closely related to soil biogeochemical processes under the disturbance of penguin or seal activities: soil C:N alteration and sufficient input of NH_4^+–N and phosphorus from animal
excrements. The results provide insights into the mechanisms how microbes drive nitrification in maritime Antarctica.

Keywords: Antarctic tundra, AOA, AOB, Marine animals, Nitrification, Nitrogen deposition
1 Introduction

Nitrification, the oxidation of ammonia into nitrate through nitrite, plays a pivotal role in the global biogeochemical cycle for nitrogen (Nunes-Alves, 2016). As the first and rate-limiting step of nitrification, ammonia oxidation (the aerobic oxidation of ammonia into nitrite) is performed by phylogenetically and physiologically distinct groups of ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) (Könneke et al., 2005; Wang et al., 2015). Only recently were the comammox, which directly oxidize ammonia to nitrate on their own, identified in the bacterial genus *Nitrospira* (Daims et al., 2015; Kessel et al., 2015). The AOA and AOB have been investigated using the *amoA* gene as a functional marker in a wide variety of environments, including soils (Di et al., 2009; Gubry-Rangin et al., 2017; Leininger et al., 2006; Ouyang et al., 2016; Shen et al., 2012), sediments (Li et al., 2015; Zheng et al., 2013), estuaries (Dang et al., 2008; Mosier et al., 2008; Santoro et al., 2011), oxic and suboxic marine layers (Baker et al., 2012; Bouskill et al., 2012), plateau permafrost (Zhang et al., 2009; Zhao et al., 2017), and in sub-arctic and arctic soil (Alves et al., 2013; Daebeler et al., 2017). Results indicated that the relative abundance and functional importance of AOA vs. AOB vary greatly in natural ecosystems. Environmental drivers, including substrate concentration, oxygen availability, pH, and salinity, might be responsible for the different AOA and AOB abundances and distribution (Alves et al., 2013; Bouskill et al., 2012; Le Roux et al., 2008; Wang et al., 2015). The abundance, diversity, and activity of ammonia-oxidizers have been explored in tundra soils of Antarctic Peninsula (Jung et al., 2011; Vergeau et al., 2007), the Antarctic Dry Valleys (Aytön et al., 2010; Magalhães et al., 2014; Richter et al., 2014), and in the Antarctic coastal waters (Kalanetra et al., 2009; Tolar et al., 2016). However, there has been limited research about the abundance and diversity of microbes.
and genes involved in the nitrogen cycle in the remote Antarctic terrestrial ecosystems. There is
still a large gap in our understanding of factors that control AOA versus AOB prominence, and
the relationships between nitrification rates and ammonia-oxidizer dynamics need to be explored
in the Antarctic.

In maritime Antarctica, a large number of sea animals, such as penguins or seals, settle on
some coastal ice-free tundra patches. Tundra vegetation including mosses, lichens, and algae,
penguin colonies, and their interactions, form a special ornithogenic tundra ecosystem (Tatur et
al., 1997). The soil biogeochemistry of an ornithogenic tundra ecosystem has become a research
hotspot under the penguin-activity disturbance (Otero et al., 2018; Riddick et al., 2012; Simas et
al., 2007; Zhu et al., 2013, 2014). Previous studies indicated that sea animals significantly affect
the tundra N and P cycles (Lindeboom et al., 1984; Simas et al., 2007; Zhu et al., 2011), and the
total N and P excreted by seabird breeders and chicks are 470 Gg N yr$^{-1}$ and 79 Gg P yr$^{-1}$ in
Antarctica and the Southern Ocean, accounting for 80% of the N and P from total global seabird
excreta (Otero et al., 2018). Uric acid is the dominant N compound in penguin guano, and during
its mineralization, different N forms, such as NH$_3$, NH$_4^+$, and NO$_3^-$, can be produced via
ammonification, nitrification, and deposition, following the changes in soil pH and the C:N ratio
(Blackall et al., 2007; Otero et al., 2018; Riddick et al., 2012). The alteration of soil
biogeochemistry under the disturbance from sea animal activities might have an impact on the
abundance and diversity of the AOA and AOB involved in the nitrogen cycle. Increased bacterial
abundance, diversity, and activity have been detected in penguin or seal colony soils (Ma et al.,
2013; Zhu et al., 2015). Penguin or seal colonies have been confirmed as strong sources for
greenhouse gas N$_2$O (Zhu et al., 2008, 2013), a by-product of microbial ammonia oxidation.
(Santoro et al., 2011). However, the effects of sea animal colonization on AOA and AOB community structures have not been thoroughly investigated in the maritime Antarctic tundra.

In the present study, we investigated the abundance, activity, and diversity of soil AOA and AOB in five tundra patches, including a penguin colony, a seal colony, the adjacent animal-lacking tundra, tundra marsh, and background tundra, where soil biogeochemical properties were subjected to the differentiating effects of sea animal activities. Our objectives were (a) to examine the abundance, diversity, and community structure of soil AOA and AOB using the *amoA* gene as a functional marker; (b) to investigate potential links between *amoA* gene abundance, AOA and AOB community structures, activity, and environmental variables; and (c) to assess the relative contribution of these two distinct ammonia-oxidizing groups to nitrification.

2 Materials and methods

2.1 Study area

The study area is located on the Fildes Peninsula and Ardley Island in the southwest of King George Island (Fig. 1), having an oceanic climate characteristics. Mean annual air temperature is about −2.5 °C, with a daily mean range from −26.6 to 11.7 °C, and mean annual precipitation is about 630 mm, mainly in the form of snow. The Fildes Peninsula (about 30 km² area) is a host to important sea animal colonies. Based on annual statistical data, the total of over 10,700 sea animals colonize this peninsula in austral summer. On the western coast are some established seal colonies including elephant seal (*Mirounga leonine*), weddell seal (*Leptonychotes weddelli*), fur seal (*Arctocephalus gazella*) and leopard seal (*Hadrurga leptonyx*) (Sun et al., 2004). Ardley Island, with an area of 2.0 km in length and 1.5 km in width, is connected with the Fildes Peninsula.
via a sand dam. This island belong to an important Ecological Reserve for penguin populations in western Antarctica. A great many of breeding penguins, including Adélie penguins (*Pygoscelis adeliae*), Gentoo penguins (*Pygoscelis papua*), and Chinstrap penguins (*Pygoscelis antarctica*), colonized on the east of this island in the austral summer. Seal excrements or penguin droppings rich in nitrogen and phosphorus were transported into local tundra soils by ice-snow melting water during the breeding period. Mosses and lichens dominate local vegetation. However, the vegetation is almost absent in penguin or seal colonies because of overmanuring and animal trampling. More detailed description about the study area can be found in Zhu et al. (2013).

### 2.2. Tundra soil collection

In the summer of 2014/2015, soil samples were collected from the following tundra patches, as illustrated in Fig. 1:

(i) Penguin colony and penguin-lacking tundra sites: The tundra on Ardley Island was categorized into three areas from the east to west according to the distance to the penguin nesting sites (i.e., the intensity of penguin activity): The eastern active penguin colony with nesting sites PTS (i.e., high penguin-activity area) where penguins have the highest density and high frequency presence during the breeding period; the adjacent penguin-lacking tundra areas, PLS (i.e., low penguin-activity areas) in the middle of Ardley Island where penguins occasionally wander and have a typically low density; and the western tundra marsh, MS, moderately far from penguin nesting sites (i.e., a slight penguin-activity area) where penguins rarely frequent the sites. In total, fourteen soil samples were collected from Ardley Island to study the effects of penguin colonization on the abundance, activity, and community structures of soil AOA and AOB.
Specifically, samples PS1–PS5 were collected sequentially from the center of the colony in the PTS. Samples PL1–PL4 and MS1–MS5 were randomly collected in the PLS and MS. (ii) The seal colony and its adjacent tundra sites, STS: These sites are on the western coast of the Fildes Peninsula. According to the distance to seal wallows (i.e., the intensity of seal activity), samples SS1–SS5 were collected in sequence to investigate the effects of seal colonization. Site SS1 was closest to the seal colony (i.e., a high seal-activity site), whereas SS5 was the farthest from the seal colony (i.e., a low seal-activity site). (iii) Background tundra sites, BS: Three soil samples were collected from an upland tundra with about 40 m a.s.l. and the distribution of no sea animal around. The tundra surface is covered with mosses or lichens with a 10–15 cm organic clay layer (Zhu et al., 2013).

At each sampling site, soil was collected aseptically using a clean scoop from the top 5–10 cm at the four corners of a 1 m² subarea, and combined into one sample. Appropriate precautions were taken to avoid cross-site or human-made contamination. Immediately after collection, each sample was divided into two portions: one was stored in sterile plastic containers at −80 °C for the analysis of the microbial community structures, and the other portion was stored at close to the in situ temperature to determine the geochemical characteristics and potential ammonia oxidation rates. All of the analyses were conducted within one month.

2.3. General analysis of soil characteristics

Soil pH was determined by mixing the soil and 1 M KCl solution (1: 3 ratio). Soil moisture was measured by oven drying at 105 °C to a constant weight. Total nitrogen (TN) and total sulfur (TS) contents in the soils were determined through a CNS analyzer (vario MACRO, Elementar,
Germany). The chemical volumetric method was used to measure soil total organic carbon (TOC). The samples were digested in Teflon tubes using HNO₃-HCl-HF-HClO₄ digestion at 190 °C, and total phosphorus (TP) was determined using ICP-OES (Perkin Elmer 2100DV, Waltham, MA, USA). The NO₃⁻-N, NO₂⁻-N, and NH₄⁺-N concentrations were determined through a continuous flow analyzer (Skalar, Netherlands) (Gao et al., 2018; Zhu et al., 2011).

2.4. Measurement of soil ammonia oxidation rate

Potential ammonia oxidation rate (PAOR) in tundra soil was determined using the chlorate inhibition method (Kurola et al., 2005; Yue, 2007). Sodium chlorate was used to inhibit NO₂⁻ from being oxidized into NO₃⁻. Briefly, 5 g fresh tundra soil was incubated in 20 ml of 1 mM phosphate-buffered saline with 1 mM of (NH₄)₂SO₄ and NaClO₃ in the dark at 15 °C. After moderately shaking for 24 h, the 5 ml of 2 M KCl was used to extract the nitrite. The optical density for the supernatant after centrifugation was determined spectrophotometrically at 540 nm. The standard curve obtained from NaNO₂ (0–2.5 μmol l⁻¹) was used to calculate the PAOR in the tundra soils.

2.5. DNA extraction and gene amplification (PCR)

Genomic DNA was extracted from 0.25 g of homogenized tundra soils using PowerSoil™ DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) as described in manufacturer’s protocol. The extracted DNA was eluted in 50 μl of elution buffer, quantified by a Nanodrop-2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), and stored at −20 °C. AOA amoA gene fragments (635 bp) were amplified using the primers Arch-amoAF (5’-STAATGGTCTGGCTTAGACG-3’) and Arch-amoAR (5’-GCGGCCATCCATCTGTATGT-3’).
(Francis et al., 2005). The amoA gene fragment (491 bp) of β-proteobacterial AOB, which represents known AOB in soil, was amplified using the primer set composed of amoA-1F (5’-GGGTTTTCTACTGGTGGT-3’) and amoA-2R (5’-CCCCTCKGSAAGCCTTCTTC-3’) (Rotthauwe et al., 1997). All PCR reactions were performed using Taq PCR Master Mix (Sangon Biotech, Shanghai, China) in a total volume of 50 μl. PCR reactions were carried out with a thermal profile of 5 min at 95 °C; 35 cycles of 94 °C for 30 s, 56 °C for AOA or 55 °C for AOB for 45 s, 72 °C for 1 min; and a final 5-min extension cycle at 72 °C (Zheng et al., 2014).

Subsequently, the amplification products were visualized by electrophoresis on 1.0 % agarose gels.

2.6. Sequencing and phylogenetic analysis

The amplification products were sent to Sangon Company (Shanghai, China) for purification, cloning and sequencing (Zheng, 2014) The sequences were edited using DNAstar (DNASTAR, Madison, WI, USA), and then aligned by muscle using the UPGMB clustering method with the ClustalX program. The sequences with 97% identity were grouped into one OTU using the Mothur Program by the furthest neighbor approach (Zheng et al., 2014). The closest reference sequences were identified at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTn tool, and phylogenetic trees were constructed by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis software (version 5.03). The sequences reported in this study have been deposited in GenBank under accession numbers MH318029 to MH318568 and MH301331 to MH302505.
2.7. Quantitative real-time PCR

The AOB and AOA amoA gene copy numbers for tundra soils were determined in triplicate using an ABI 7500 Sequence Detection System (Applied Biosystems). The specific details were given by zheng et al. (2014). The strong linear inverse relationship confirmed the consistency of the qPCR assay between the threshold cycle and the log value of gene copy numbers ($R^2 = 0.999$ for AOB; $R^2 = 0.997$ for AOA). The amplification efficiencies for AOA and AOB were 99.8 % and 90.4 %, respectively. Melting curve analysis had only one observable peak at a melting temperature (Tm) (84.9 °C for AOA and 89.6 °C for AOB) (Supplementary Fig. S3). Negative controls were subjected to exclude any possible carryover or contamination in all experiments.

2.8. Statistical analysis

The Shannon–Weiner Index, Simpson Index and the richness estimator Chao 1 were calculated by the Mothur program (version 1.23.0). The coverage was the percentage of the number of observed OTUs divided by the Chao 1 (Supplementary Table S2). The Kruskal–Wallis test and Wilcoxon signed rank test were conducted for the comparison between amoA gene abundance and PAOR from five tundra patches using SPSS Statistics 17 (IBM Corp, Armonk, NY, USA). The relationships between the ammonia-oxidizer community structure and environmental variables were explored using canonical correspondence analysis (CCA) in the software Canoco for windows (version 4.5; Microcomputer Power, Ithaca, NY, USA), because the maximum gradient length of both AOA and β-AOB was longer than four SD (AOA: 4.406; AOB: 18.326). All environmental parameter values were transformed into $\ln(x+1)$ before statistical analyses. OTU richness (defined at 3% distance) served as the species input and several simulations of manual
forward selection were performed with 499 Monte Carlo permutations to build the optimal models.

The scaling in the final CCA biplots was focused on inter-sample relations. Correlations between ammonia-oxidizer gene abundance, diversity, PAOR, and the AOB/AOA ratio with environmental variables were explored using redundancy analysis (RDA), because the maximum gradient length was shorter than three SD (AOA: 0.09; AOB: 0.088; PAOR and AOB/AOA: 1.105).

3 Results

3.1. Soil chemistry and sea animal activities

Overall, almost all the tundra soils were slightly acidic, with a mean pH range of 5.3–6.6. Penguin and seal colony tundra soils, PTS and STS, had lower TOC contents and C:N ratios than the animal-lacking tundra soils (PLS), tundra marsh soils (MS), and background tundra soils (BS).

As expected, soil nutrient levels (TN, TP, TS, and NH$_4^+$–N) were higher in PTS, STS, PLS, and MS than in BS (Table 1). Soil NH$_4^+$–N contents were 1–2 orders of magnitude higher in PTS and STS than in PLS, MS, and BS, with the means of 176.9 and 137.6 mg NH$_4^+$-N kg$^{-1}$, respectively.

The highest NO$_3^-$-N contents occurred in STS. Phosphorus levels were significantly greater ($p < 0.05$) in PTS (10.6–32.9 mg g$^{-1}$) than in the other types of tundra soils (mean < 6.0 mg g$^{-1}$). In the seal colony tundra, soil TOC, TN, TP, TS, and NH$_4^+$-N levels decreased with the distance from the seal wallow. Likewise, soil TP, TS, and NH$_4^+$-N levels decreased from the eastern penguin nesting sites to the western tundra marsh. Sea animal activities altered the local soil biogeochemical properties through the deposition of their excreta, leading to generally low C:N ratios and a marked increase in soil NH$_4^+$-N and TP contents. Therefore, the soil TP and NH$_4^+$-N
levels and the distance from seal wallows and penguin nesting sites could be used to assess the intensity of seal or penguin activities.

3.2. Gene abundances under sea animal colonization

The abundance of the AOB amoA gene was significantly higher (by approximately 2–4 orders of magnitude) than that of the AOA amoA gene (Wilcoxon test, n = 22, P = 0.002) in the penguin and seal colony and their adjacent tundra soils, PTS, STS, and PLS. However, the abundances of the amoA gene were similar in the MS and BS soils (Fig. 2). Overall, the abundances of AOB and AOA amoA genes were significantly negatively correlated (r = -0.90, P = 0.037) across all the tundra sites. The archaeal amoA gene showed a heterogeneous distribution among the different tundra patches. AOA amoA gene were two orders of magnitude lower in PTS and STS relative to those in BS and MS. The maximal AOA amoA gene abundance appeared in BS, followed by MS and PLS, whereas the PTS and STS soils had the lowest archaeal amoA gene abundances. Soil AOA amoA gene abundances were significantly increased with decreasing animal activity intensity (i.e., the distance from eastern penguin nesting sites PS1–PS5 to western tundra marsh MS1–MS5, and from seal wallow site SS1 to the background tundra sites) (Fig. 3).

Unlike the AOA amoA genes, AOB amoA gene abundances showed the opposite distribution pattern. The AOB amoA gene abundances were significantly higher (by approximately 2–3 orders of magnitude) in PTS and STS compared with those in MS and BS (Fig. 2). The soil AOB amoA gene abundances increased significantly with increasing animal activities (i.e. the distance from eastern penguin nesting sites and from the seal wallow) (Fig. 3). The ratios of AOB to AOA amoA copy numbers were strongly affected by animal activities, and were much higher in PTS and STS.
than in PLS, MS, and BS (Kruskal–Wallis test, $\chi^2 = 18.2, P = 0.01$). Overall, penguin or seal activities increases the abundance of soil AOB amoA genes, but reduced the abundance of AOA amoA genes, leading to very large ratios ($1.5 \times 10^2$ to $3.2 \times 10^4$) of AOB to AOA amoA copy numbers in PTS and STS. However, the ratios varied only from 0.1 to 7.2 in BS and MS.

3.3 Potential ammonia oxidation rates under sea animal colonization

Potential ammonia oxidation rates (PAORs) ranged from 8.9 to 138.8 μg N kg$^{-1}$ h$^{-1}$ in all the soil samples (Table 1). The PAOR was significantly higher in STS (mean 76.1 μg N kg$^{-1}$ h$^{-1}$) and PTS (mean 64.7 μg N kg$^{-1}$ h$^{-1}$) than in PLS, MS, and BS (mean 12.0–21.8 μg N kg$^{-1}$ h$^{-1}$; Kruskal–Wallis test, $\chi^2 = 11.6, P = 0.02$). The PAOR followed the distribution changes of AOB amoA gene abundances, but showed the opposite trend to the AOA amoA gene abundances (Fig. 2). A significant positive correlation ($r^2 = 0.77, P < 0.001$) was observed between the PAOR and the AOB amoA gene abundance when the data from all the tundra patches were combined, whereas no correlation occurred between PAOR and AOA amoA gene abundance (Fig. 4). Therefore, the AOB populations might contribute more to the PAOR than the AOA populations in the study area. Interestingly, the PAOR greatly increased with penguin or seal activity intensity, and the greatest rates occurred at the sites nearest the penguin nests ($88.8 \pm 2.7$ μg N kg$^{-1}$ h$^{-1}$) and seal wallows ($138.8 \pm 0.8$ μg N kg$^{-1}$ h$^{-1}$) (Fig. 3).

3.4. Community structure of AOA and AOB under sea animal colonization

The PCR products were insufficient to construct the clone libraries for the AOA amoA gene from STS and PTS because of the low AOA abundance in the soils, as was the case with the AOB amoA gene from MS and BS. Overall, 10 AOA and 14 AOB amoA gene clone libraries were
successfully constructed. 543 AOA sequences and 1175 AOB quality sequences were generated from the respective sites. Within each individual site, 1–6 AOA OTUs and 6–15 AOB OTUs were identified, as defined by < 3% divergence in nucleotides. The AOA and AOB OTU numbers for each library are presented in Table S1. These numbers might be higher if more clones were sequenced, based on the rarefaction curves (Fig. S1 and Fig. S2). The diversity of the AOB amoA was generally higher than that of AOA amoA, based on the indices of Shannon–Wiener and Simpson. Specifically, the AOA amoA gene had higher diversity in PLS and MS than in BS. The AOB amoA gene showed higher diversity in STS and PTS compared with that in adjacent animal-lacking tundra soils.

The 543 AOA amoA gene sequences had 76–100% sequence similarity to each other, and 95–100% identity with the corresponding top hit amoA sequences deposited in GenBank. Phylogenetic analysis showed that the AOA amoA sequences could grouped into 16 unique OTUs, representing 100% of all the AOA amoA OTUs identified, and were affiliated with two Nitrososphaera clusters (Fig. 5a): Cluster I had 11 OTUs and 264 clones, and 57.9% of AOA amoA sequences were from PLS, 41.3% from STS, and only 0.8% from MS. In Cluster II, there are five unique OTUs and 279 clones, and 58.8% of them were from BS, 38.3% from MS, and only 2.9% from PLS. Almost all the AOA phylotypes retrieved from PLS and STS were related to Nitrososphaera cluster I, whereas the AOA phylotypes retrieved from MS and BS were distributed in cluster II (Fig. 6). Seal or penguin activities led to the predominant existence of AOA phylotypes related to cluster I, but very low relative abundances in AOA phylotypes related to cluster II, which were almost completely excluded in STS and PLS. Almost all AOA phylotypes
in BS and MS were related to *Nitrososphaera* cluster II, whereas the relative abundances of AOA phylotypes related to cluster I were very low or undetectable.

The 1175 AOB *amoA* gene sequences shared 87–100% sequence identity to each other, and 93–100% identity with the closest matched GenBank sequences. Phylogenetic analysis showed that the AOB *amoA* sequences could be grouped into 38 unique OTUs, representing 58.5% of all the AOB *amoA* OTUs identified, and these *amoA* sequences were grouped into four clusters according to the evolutionary distance of the phylogenetic tree with known sequences from AOBs in the *Nitrosospira* genera (Fig. 5b). Cluster I had 11 OTUs and 226 clones, and 67.7% of AOB *amoA* sequences were from PTS, 23.5% from STS, 8.4% from PLS, and only 0.4% from MS. There are 17 unique OTUs and 521 clones in clusters II and III. The sources of the OTUs in cluster II were similar to those of cluster I, with 69.8% from PTS, 29.9% from STS, and 0.3% from PLS. For cluster III, 79.2% of the sequences were from PLS, 19.8% from STS, and 1.0% from MS. Cluster IV had nine unique OTUs and 370 clones from PLS (50.0%), STS (36.8%) and MS (13.2%), respectively. Of all the AOB phylotypes retrieved from PTS were related to dominant *Nitrosospira* clusters I and II, whereas AOB phylotypes related to cluster III and IV were completely excluded because of strong penguin activity (Fig. 6). The AOB phylotypes retrieved from STS were distributed in clusters I, II, III, and IV (16–38% for each cluster). Almost all the AOB phylotypes retrieved from PLS and MS were related to *Nitrosospira* clusters III and IV.

### 3.5. Relationships of the ammonia-oxidizer community structure with environmental variables

The relationships of the AOA and AOB communities with environmental variables were analyzed using CCA. The environmental variables explained 58.4% of the total variance in the
AOA *amoA* genotype compositions, and 66.8% of the cumulative variance of the genotype-environment relationships in the first two CCA dimensions (Fig. 7a). Overall, the AOA community structures significantly correlated with C:N, TOC, and NO$_3^-$-N in tundra soils (Table 2), and the combination of the three factors explained 60.3% of the variation. Although other environmental parameters, including TP, pH, and NH$_4^+$-N were not statistically significant (*P* > 0.05), these variables additionally explained 26.5% of the variation. The AOA richness and phylotypes were evidently inhibited in STS and PLS because seal or penguin activities. However, high soil C:N and TOC concentrations increased the AOA richness and phylotypes in MS and BS.

As illustrated in Fig. 7b, the first two dimensions explained 26.6% of the total variance in the AOB compositions, and 54.3% of the cumulative variance of the AOB genotype-environment relationships. The composition and distribution of AOB communities correlated significantly with NH$_4^+$-N and C:N ratios, and the two factors combined yielded 21.9% of total CCA explanatory power. The others including TP, NO$_3^-$-N and pH accounted for 27.1% of the variance. Penguin or seal activities significantly increased the AOB richness and phylotypes in STS and PTS through higher NH$_4^+$-N and P input from sea animal excrement, whereas AOB richness and phylotypes were closely related to the soil C:N in PLS and MS.

Correlations among *amoA* gene abundance, diversity, PAOR, and the ratios of AOB:AOA abundance with environmental variables were examined via Redundancy Analysis (RDA) (Fig. 8). The AOA *amoA* gene abundance and diversity were positively related to the C:N ratio (*P* = 0.002), and negatively correlated with NH$_4^+$-N (*P* = 0.004). Two factors combine yielded 63.5% of the total RDA explanatory power (Table S2). Higher soil C:N increased the AOA abundance and diversity in BS and MS, but higher NH$_4^+$-N input inhibited their abundance and diversity in
PLS and STS because of penguin or seal activities. Significant correlations were obtained between AOB \textit{amoA} gene abundance, diversity, and environmental factors including the C:N ratio (P = 0.004), TOC (P = 0.012), and NH$_4^+$-N (P = 0.05). These three factors combined yielded 73.2% of the total explanatory power (Table S3). The ratios of AOB to AOA and PAOR showed positive correlations with NH$_4^+$-N (P = 0.002), TP (P = 0.046), and TS (P = 0.030), but negative correlations with the C:N ratio (P = 0.002) and TOC (P = 0.048). These factors explained 87.5% of the variation (Table S4). Compared with those in BS and MS, penguin or seal activities significantly increased the AOB \textit{amoA} gene abundance, diversity, PAOR, and the ratios of AOB to AOA in STS, PTS, and PLS because of the increase in NH$_4^+$-N and TP input from animal excrement.

4 Discussion

4.1. Effects of sea animal colonization on AOA and AOB abundances

In this study, soil AOA \textit{amoA} gene abundances were two orders of magnitude lower in PTS and STS relative to BS and MS; however, AOB \textit{amoA} gene abundances were approximately 2–3 orders of magnitude higher in PTS and STS than in MS and BS, indicating that sea animal activities increased the AOB population size, but inhibited AOA abundances in tundra soils (Fig. 2 and Fig. 3). Overall, the archael \textit{amoA} gene abundances obtained here were similar to the abundance range reported in the soils of the Antarctic Dry Valleys and arctic tundra soils; however, the bacterial \textit{amoA} gene abundances were two to three orders of magnitude higher in PTS and STS than in Antarctic Dry Valleys (Alves et al., 2013; Magalhães et al., 2014). In contrast to previous studies indicating that AOA were more abundant than AOB in some terrestrial or marine
ecosystems (Beman et al., 2008; Lam et al., 2007; Wuchter et al., 2006; Yao et al., 2011), and in soils from Antarctic Peninsula (Jung et al., 2011), our qPCR estimates showed that the bacterial *amoA* copy numbers were much greater than those of archael *amoA* in PTS, STS and PLS because of sea animal activities. However, their abundances were very close to each other in BS and MS. The ratios of AOB to AOA abundance were strongly affected by sea animal activities. A shift in the relative abundance of AOA and AOB recorded previously for the Antarctic Dry Valleys, with a greater abundance of AOB compared with that of AOA for Battleship Promontory and Miers Valley, and the reverse for Upper Wright Valley and Beacon Valley (Magalhães et al., 2014). The results for PTS, STS, and PLS are also in agreement with those detected in subglacial soils (Boyd et al., 2011).

The ratios of AOB to AOA showed significant positive correlations with NH$_4^+$-N, TP, and TS when all the data were combined in the five tundra patches (Fig. 8). This suggested that NH$_4^+$-N, TP, and TS are key factors when bacterial *amoA* genes are much more abundant than archael *amoA* genes. In Antarctica, the productivity of terrestrial ecosystems is strongly limited because of the extremely low nitrogen levels (Park et al., 2007). However, the physiochemical properties for tundra soils were strongly influenced by the deposition of penguin or seal excreta under effects of local microbes (Tatur et al., 1997). Sea animals provide considerable external N inputs for their colony soils and adjacent tundra soils through direct input of their excreta and atmospheric deposition via ammonia volatilization (Lindeboom, 1984; Sun et al., 2002; Blackall et al., 2007; Zhu et al., 2011; Riddick et al., 2012). Like ammonium, P and S are typical elements in penguin guano, and they have been used to indicate penguin activity intensity (Sun et al., 2000). Significantly elevated NH$_4^+$–N and TP concentrations occurred in PTS and PLS compared with
those in BS (Table 1). These conditions may be beneficial for nitrification, allowing high abundance and diversity of bacterial amoA, which explains the strong correlations between AOB abundances and NH$_4^+$–N, TP, and TS in the sea animal colony soils (Fig. 8). This is agreed with the high bacterial diversity and abundance previously documented in penguin or seal colony soils and ornithogenic sediments (Ma et al., 2013; Zhu et al., 2015).

The AOA abundance and diversity showed a positive correlation with C:N in tundra patches, but a significant negative correlation with NH$_4^+$-N levels (Fig. 8). AOA might better adapt to low NH$_4^+$ and oligotrophic environments because the half-saturation constant for ammonia oxidation by *Thaumarchaeota* is lower than that by AOB (Martens-Habbema et al., 2009). High NH$_4^+$-N concentrations might partially inhibit AOA populations (Hatzenpichler et al., 2008). This result is similar to that reported for some agricultural soils with increased fertilization, and grassland soils with increased grazing (Fan et al., 2011; Prosser and Nicol, 2012; Pan et al., 2018), supporting the conclusion that AOA and AOB generally inhabit different niches in soil, distinguished by the NH$_4^+$ concentration and availability (Verhamme et al., 2011; Wessén et al., 2011).

**4.2. Effects of sea animal colonization on soil ammonia oxidation rates**

In this study, PAOR ranged from 9 to 139 μg N kg$^{-1}$ h$^{-1}$, which was lower than nitrification rates measured in most agricultural soils (83–1875 μg N Kg$^{-1}$ h$^{-1}$) (Fan et al., 2011; Ouyang et al., 2016; Daebeler et al., 2017). One reason might be the selection of a 15 °C incubation temperature, which is lower than the incubation temperatures used in other studies. Generally, the gross nitrification rate and amoA abundance increased significantly when the incubation temperature was higher than 15 °C (Daebeler et al., 2017; Zhao et al., 2014). Notably, comammox
Nitrospira may actually compete with ammonia oxidizers for ammonium, after which comammox oxidize ammonia to nitrate on their own via a one-step process (Daims et al., 2015; van Kessel et al., 2015). In this study, the method of measuring nitrification rates did not include the activity of these organisms because sodium chlorate was used to prevent NO$_2^-$ from being oxidized to NO$_3^-$, whereas other methods likely capture the comammox activity (Santoro, 2016). Our measurements indicated that there were significant differences in the PAOR across different tundra patches ($P = 0.02$), and the PAORs in STS and PTS were about 10 times higher than those in BS and MS. A significant correlation was observed between the PAOR and NH$_4^+–N$, TP, and sulfur (Fig. 8). Overall, ammonia oxidation activity was modulated by soil biogeochemical processes under the disturbance of sea animal activities: sufficient input of the nutrients NH$_4^+–N$, TP, and TS from sea animal excreta.

The gene abundance of AOB amoA was markedly higher that of AOA amoA, and AOA found it difficult to tolerate the high ammonium environment in PTS, STS, and PLS, indicating that AOB might play a more important role in nitrification. In agreement with these results, AOB dominated nitrification in the areas where it was easy to achieve nitrogen input, whereas the relative contribution of AOA to nitrification was higher in the areas where the ammonium concentration remained low (Fan et al., 2011; Sterngren et al., 2015). Moreover, the cell-specific activity for AOB was 10 times higher than that for AOA due to the bigger cell size of AOB (Hatzenpichler et al., 2012; Prosser and Nicol, 2012). Therefore, AOB might play a more important role in nitrification in STS, PTS, and PLS compared with that in BS and MS.
In addition, AOA might play a role that cannot be ignored in MS and BS, just like the prevalence of AOA among ammonia-oxidizers in Arctic soils (Alves et al., 2013; Daebeler et al., 2017). AOB groups were mostly undetectable in the analysis of MS and BS. Although unknown γ-AOB groups might not have been detected, the primer set used here covers the β-AOB groups typically found in soils (Alves et al., 2013). The BS and MS were covered with lush tundra plants and were rich in organic carbon (Table 1), which has been shown to favor AOA because their substrates can be provided through the mineralization of soil organic matter (Stopnišek et al., 2010; Habteselassie et al., 2013).

4.3. Effects of sea animal colonization on genotypic diversity of soil AOA and AOB

In this study, distinct AOA communities appear to inhabit different types of tundra patches, depending on sea animal activities (Fig. 5). It was difficult to amplify the AOA amoA gene from STS and PTS, whereas a high diversity of AOA amoA genes was observed in PLS, MS and BS. Phylogenetic analysis indicated that the AOA amoA sequences in Cluster I were from PLS and tundra soils close to seal wallows, while the sequences in Cluster II were from BS and MS (Fig. 6). AOA in most extreme environments have lower levels of microbial diversity than benign ecosystems because of the requirement for specific physiological adaptations, which allow organisms to exploit the combination of physical and biochemical stressors (Cowan et al., 2015). Cluster I found in the PLS might represent AOA adapting to survival in the presence of relatively high soil nutrients, for which the presence of the amoA gene represents either secondary metabolism or an ancestral remnant no longer active because of the high AOB abundance in these areas. Detected OTUs in Cluster I had their closest matches mainly from the hyper-arid soils of
Antarctic dry valleys (Magalhães et al., 2014), wetland soils (Zheng et al., 2014), alpine meadow soils (Zhao et al., 2017), and some agricultural soils (Glaser et al., 2010). Cluster II were more prevalent in BS and MS, probably because of their stronger adaptation to barren soil environments.

In cluster II, the sequences were affiliated with sequences recovered from cold environments, including the soils of Tibetan Plateau (Zhang et al., 2017) and Icelandic grassland soils (Lam et al., 2009). The compositions of soil AOA populations are likely not to be explained by single physicochemical properties, and their community structures significantly correlated with tundra soil C:N, TOC, and NO$_3^-$-N, which was consistent with previous studies (Glaser et al., 2010; Wessén et al., 2011).

The AOB $amoA$ gene generally had a higher diversity than AOA, similar to results in the Antarctic Dry Valley soils (Magalhães et al., 2014). A high diversity of AOB $amoA$ gene occurred in STS, PTS and PLS compared to BS, indicating that penguin or seal activities had important effects on AOB genotypic diversity. According to the evolutionary distance of the phylogenetic tree, AOB $amoA$ sequences were grouped into four clusters with known sequences from the Nitrosospira genera, and they are in the lineages of Nitrosospira sp. En13 (EF175097), Nitrosospira sp. LT1FMf (AY189144), Nitrosospira sp. EnI299 (EF175100), Nitrosospira sp. III7 (AY123829), and Nitrosospira sp. Wyke8 (EF175099) (Fig. 5b). The sequences in clusters I and II were mainly from PTS and STS, and the detected OTUs in Cluster I had their closest matches from mixed community culture systems, meadow to forest transect in Oregon Cascade Mountains (Mintie et al., 2003), and Dutch agricultural soils and reservoir sediments (Silva et al., 2012). For Clusters III and IV, the sequences were predominantly from PLS and STS, and they were affiliated with sequences recovered from high altitude wetland (Shan et al., 2014). Previous studies have
shown that multiple environmental factors affected the AOB communities (Dang et al., 2008; Mosier and Francis, 2008). In this study, the NH$_4^+$-N concentrations seemed to be the most important factors influencing the AOB community structure, which was in accordance with the results from different environments (Bouskill et al., 2012; Jung et al., 2011; Li et al., 2015). Moreover, the C:N ratio and TP also affected the AOB amoA community compositions (Zheng et al., 2013). Therefore, the AOB community compositions were impacted by the biogeochemical factors related to sea animal activities, such as sufficient supply of the nutrients NH$_4^+$–N and TP from sea animal excreta.

5 Conclusions

The findings of this study concerning the abundance, activity, and diversity of tundra soil AOA and AOB provide insights into microbial mechanisms driving nitrification in maritime Antarctica. We confirmed the presence of AOA and AOB amoA genes in five different tundra patches, and demonstrated that the spatial distribution heterogeneities of the tundra soil AOA and AOB communities were driven by penguin or seal activities. The soil AOB amoA copy numbers were generally higher than the AOA amoA copy numbers, following the higher PAOR in penguin or seal colonies and their adjacent tundra, compared with that in the background tundra and marsh tundra, which are moderately far away from the animal colonies. Penguin or seal activities resulted in significant shift of soil AOA and AOB community compositions. The diversity of the AOB amoA gene was greater in STS and PTS than in PLS and MS, and the majority of the AOB sequences were closely related to Nitrosospira-like sequences. The archaeal amoA gene had higher diversity in STS, PLS, and MS than in BS, and they were associated with sequences
recovered from barren soils. Soil AOB and AOA abundances, and their community compositions, were related to soil biogeochemical processes under the disturbance of sea animal activities, such as soil C:N alteration, and a sufficient supply of the nutrients NH$_4^+$–N, N and P from animal excreta. Overall, this study significantly enhanced the understanding of ammonia-oxidizing microbial communities in tundra environment of maritime Antarctica.

Acknowledgments

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References


Table 1. Soil properties, potential ammonia oxidation rates, ammonia oxidizer populations, and diversity for the soil samples (n = 28) that span a penguin colony, a seal colony, and their adjacent animal-lacking tundra across Ardley Island and the Filde Peninsula in maritime Antarctica.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>pH</th>
<th>Moisture (%)</th>
<th>TOC (%)</th>
<th>Nitrogen (%)</th>
<th>Sulfur (%)</th>
<th>TP (mg/g)</th>
<th>NH₄-N (mg/Kg)</th>
<th>NO₂-N (mg/Kg)</th>
<th>NO₃-N (mg/Kg)</th>
<th>PAOR (µg N Kg⁻¹ h⁻¹)</th>
<th>AOA (copies g⁻¹)</th>
<th>AOB (copies g⁻¹)</th>
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<tbody>
<tr>
<td>Seal colony tundra soils (STS)</td>
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<tr>
<td>SS1</td>
<td>4.8</td>
<td>0.31</td>
<td>4.88</td>
<td>1.21</td>
<td>0.34</td>
<td>3.6</td>
<td>650.9</td>
<td>4.6</td>
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<td>138.8±0.8</td>
<td>1.79×10⁵</td>
<td>9.22×10⁶</td>
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<td>SS2</td>
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<td>0.33</td>
<td>6.62</td>
<td>1.69</td>
<td>0.48</td>
<td>5.0</td>
<td>17.7</td>
<td>19.1</td>
<td>0.7</td>
<td>115.3±15.5</td>
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<td>5.92×10⁵</td>
</tr>
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<td>0.09</td>
<td>ND</td>
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<td>17.9</td>
<td>61.7</td>
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<td>1.2</td>
<td>6.0</td>
<td>12.1</td>
<td>ND</td>
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<td>5.53×10⁴</td>
<td>2.57×10⁶</td>
</tr>
<tr>
<td>SS5</td>
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<td>0.27</td>
<td>1.16</td>
<td>0.13</td>
<td>0.07</td>
<td>0.8</td>
<td>1.1</td>
<td>13.9</td>
<td>ND</td>
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<td>3.03×10⁷</td>
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<td>2.74±1.13a</td>
<td>0.65±0.30a</td>
<td>0.24±0.08ab</td>
<td>2.4±0.7a</td>
<td>137.6±114.8a</td>
<td>22.3±49.1a</td>
<td>0.3±0.12a</td>
<td>76.1±21.4a</td>
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<td>(4.0±1.4)×10⁵ab</td>
</tr>
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<td>Active penguin colony tundra soils (PTS) along the coast on Ardley Island</td>
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<td>8.27±1.44bc</td>
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<td>19.6±3.6b</td>
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<td>(4.8±0.7)×10⁵bc</td>
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<td>The middle penguin-lacking tundra soils (PLS) on Ardley Island</td>
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<td>2.39×10^6</td>
<td>1.33×10^5</td>
<td>4.93×10^4</td>
<td>3.80×10^5</td>
<td>2.3×10^5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Background tundra soils (BS) on the upland of the Fildes Peninsula |
|-------------------|-----|-----|-----|-----|-----|---------|
| BS1               | 5.3 | 5.6 | 5.3 | 5.3 | 5.3 | 5.4±0.1 |
| EC                | 0.16 | 0.18 | 0.20 | 0.20 | 0.20 | 0.35±0.01 |
| CEC               | 16.82| 18.12| 17.55| 18.12| 17.55| 17.50±0.31 |
| P                 | 0.48 | 0.51 | 0.43 | 0.51 | 0.43 | 0.47±0.02 |
| Organics          | 0.12 | 0.12 | 0.08 | 0.08 | 0.08 | 0.08±0.02 |
| Alkali            | 2.4 | 2.4 | 1.9 | 1.9 | 3.0 | 2.5±0.3 |
| Exchangeable Alkaline| 1.1 | 0.7 | 1.2 | 1.2 | 1.2 | 2.3±0.1 |
| Reactive Ca       | 23.6| 16.4 | 16.4 | 16.4 | 16.4 | 16.7±2.0 |
| Reactive Mg | 0.5 | 0.5 | 0.5 | 0.5 | 0.6 | 0.5±0.1 |
| Reactive Mg/Ca | 12.8±1.5 | 17.6±0.5 | 17.6±0.5 | 11.1±0.8 | 11.1±0.8 | 13.8±1.6 |
| Reactive Mg/Ca | 4.33×10^6 | 7.94×10^6 | 7.94×10^6 | 1.56×10^7 | 1.56×10^7 | 8.3±2.7×10^6 |
| Reactive Mg/Ca | 2.16×10^7 | 2.39×10^6 | 2.39×10^6 | 1.11×10^7 | 1.11×10^7 | 1.2±0.5×10^7 |

The western tundra marsh soils (MS) on Ardley Island
Table 2. Individual and combined contributions of soil biogeochemical properties to the AOA and AOB community structures in tundra patches.

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>F</th>
<th>P</th>
<th>Individual contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/N</td>
<td>2.815</td>
<td>0.014</td>
<td>17.7%</td>
</tr>
<tr>
<td>TOC</td>
<td>2.337</td>
<td>0.018</td>
<td>9.7%</td>
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<tr>
<td>NO$_3^-$</td>
<td>2.165</td>
<td>0.034</td>
<td>8.3%</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>0.983</td>
<td>0.466</td>
<td>9.3%</td>
</tr>
<tr>
<td>TP</td>
<td>1.012</td>
<td>0.442</td>
<td>4.6%</td>
</tr>
<tr>
<td>pH</td>
<td>1.653</td>
<td>0.094</td>
<td>4.5%</td>
</tr>
<tr>
<td>Combined effect of all factors</td>
<td></td>
<td></td>
<td>87.4%</td>
</tr>
<tr>
<td>AOB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/N</td>
<td>1.844</td>
<td>0.002</td>
<td>6.1%</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>1.823</td>
<td>0.002</td>
<td>6.9%</td>
</tr>
<tr>
<td>TP</td>
<td>1.39</td>
<td>0.078</td>
<td>11.6%</td>
</tr>
<tr>
<td>pH</td>
<td>1.383</td>
<td>0.066</td>
<td>9.1%</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>1.161</td>
<td>0.258</td>
<td>10.7%</td>
</tr>
<tr>
<td>Combined effect of all factors</td>
<td></td>
<td></td>
<td>48.9%</td>
</tr>
</tbody>
</table>
Figure 1. Study area and soil sampling sites. Panel (a), the red dot indicates the location of the investigation area in maritime Antarctica. Panel (b), location of the sampling sites on the Fildes Peninsula. The sampling soils from tundra patches included the active seal colony tundra soils STS (SS1–5) in the western coast of the Fildes Peninsula, and the background tundra soils on the upland areas (BS1–3). Panel (c), the location of the sampling sites on Ardley Island. The sampling soils from tundra patches included the western tundra marsh soils (MS1–5), the eastern active penguin colony tundra soils PTS (PS1–5) and the adjacent penguin-lacking tundra soils PLS (PL1–4). Note: The map was drawn using CorelDRAW X7 software (http://www.corel.com/cn/).
Figure 2. Comparisons of soil potential ammonia oxidation rates (PAOR) (a); AOA and AOB amoA gene copy numbers (b); and log ratio of AOB: AOA abundances (c); between five tundra patches. The error bars indicate standard deviations of the means.
Figure 3. Effects of penguin or seal activity on potential ammonia oxidation rates (PAORs), and AOA and AOB amoA gene copy numbers in tundra soils. (a) Penguin colonies and their adjacent tundra; (b) Seal colonies and their adjacent tundra. The error bars of potential ammonia oxidation rates indicate the standard deviations of triplicate incubations, whereas the error bars of the amoA copy numbers indicate standard deviations of triplicate real-time PCR assays.
Figure 4. Correlation between potential ammonia oxidation rates (POARs) and AOA and AOB amoA gene copy numbers in tundra soils of maritime Antarctica.
Figure 5. Neighbor-joining phylogenetic tree of AOA amoA (a) and AOB amoA (b). The phylogeny is based on nucleotide sequences. Bootstrap values ≥ 50% (of 1000 iterations) are shown near the nodes. GenBank accession numbers are shown for sequences from other studies. OTUs were defined at 97% similarity. Numbers in parentheses following each OTU indicate the number of sequences recovered from each sampling site.
Figure 6. Relative abundance of partial AOA (a) and AOB (b) sequences retrieved from five tundra patch soils subjected to different effects of sea animal activities, as related to different *Nitrososphaera* or *Nitrosospira* clusters.
Figure 7. Canonical correspondence analysis (CCA) ordination plots for the relationship between the AOA and AOB community structures with environmental variables. The circles with different colors represent the various sampling sites. The size of the circles corresponds to the OTU richness in individual samples. The black triangles represent amoA phylotypes. Environmental variables are represented by red arrows. The percentage of species-environment relation variance explained by the two principal canonical axes is represented close to the axes.
**Figure 8.** Redundancy analysis ordination (RDA) plots for the relationships between copy number and Shannon's diversity index of AOA *amoA* genes and environmental variables (a), between copy number and Shannon's diversity index of AOB *amoA* genes and environmental variables (b), and between the PAOR, ratio of AOB/AOA *amoA*, and environmental variables (c). Gene copy log values for AOA, AOB, and log ratios of AOB/AOA *amoA* are represented as circles whose diameters are scaled linearly to the magnitude of the value. In the RDA ordination diagram, the angle and length of the arrow relative to a given axis reveals the extent of the correlation between the variables and the canonical axis (environmental gradient). SHDI indicates Shannon's diversity index.